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Dietary Supplementation of Solid-state Fermented Yellow Mealworm (*Tenebrio molitor*) Larvae Meal Enriched by *Lactobacillus sp.* in Guppy (*Poecilia reticulata*)

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ABSTRACT

The current study examined the dietary solid-state fermented yellow mealworm (*Tenebrio molitor*) larvae meal inclusion on growth performance, gut microbiota, body composition, liver and intestinal histology and histomorphometric parameters in the guppy (*Poecilia reticulata*) for 84 days. Guppies were fed diets included with no supplementation (C); 4 g/kg yellow mealworm larva meal (G1), 4 g/kg solid-state fermented with *Lactobacillus brevis* yellow mealworm larvae meal (G2), 4 g/kg solid-state fermented with *Lactobacillus plantarum* yellow mealworm larvae meal (G3), the combination of 2 g/kg solid-state fermented with *L. brevis* plus 2 g/kg solid-state fermented with *L. plantarum* yellow mealworm larvae meal (G4). For female guppies, the growth performance of the G4 group clearly differed from all groups with the synergistic effect of solid-state fermented with *L. plantarum* plus *L. brevis*. In male guppies, G3 and G4 groups showed the highest growth performance values among all groups. The intestinal microbiota of guppies was clearly varied with supplementation groups. Fusobacteria was the most abundant phylum in C, G1, G2 and G3 groups. However, Proteobacteria showed the most intensity in the G4 group. Intestinal villus height, width and surface area were positively affected in solid-state fermented yellow mealworm larvae meal supplementation groups, reaching higher values in G3 and G4 groups. In conclusion, solid-state fermented yellow mealworm larvae meal via 2 g/kg *L. plantarum* plus 2 g/kg *L. brevis* can improve growth performance by modulating the gut microbiota of guppies.

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Introduction

Although most of the global aquaculture production is devoted to food products, ornamental fish production continues to expand as one of many countries' crucial products of the aquaculture industry (Rahmati-Holasoo et al., 2022). The ornamental fish sector mostly depends on aquaculture in the supply of feed raw materials. In recent years, the rapid growth of aquaculture production has caused concern in the feed industry due to limited resources (Gu et al., 2022). Moreover, the importance of improving feed formulations with functional ingredients has emerged as one of the most critical factors for all aspects of the sustainability of the sector (Ghamkhar & Hicks, 2020). At this point, developing production strategies that will promote the healthy growth of cultured fish in the diet is expressed as an effective solution for the efficient use of resources (Rohani et al., 2021). As in all aquaculture production areas, approaches to the sustainable use of feed resources in the ornamental fish sector draw attention (Sultana et al., 2022). The aquarium hobby has gained value as a new activity for most people who stay home during the coronavirus pandemic (Hood et al., 2021). Also, the pandemic has revealed that countries should produce some aquarium species with local possibilities and the need for feed additives to increase the diet quality. Feed additives can positively affect the health of the fish's digestive system (Dawood et al., 2018). In recent years, regulating the gut microbiota in fish's digestive system has been among the priority studies to improve healthy growth in cultured species (Tan et al., 2019; Terova et al., 2019; Deng et al., 2022). Common methods to regulate the gut microbial community include enrichment feed formulations with functional feed additives (FFAs) such as

probiotics, prebiotics, and synbiotics. FFAs are a promising alternative growth promoter for fish performance (Benzertiha et al., 2020). In addition, previous studies emphasized that solid-state fermented feed additives improved the gut microbiota and could be potentially functional for fish species (Zhang et al., 2021; Yang et al., 2022). Also, feed efficiency and growth performance can be increased by reducing feed costs in the aquatic environment with solid-state fermented feed additives (Bowyer et al., 2020; Davies et al., 2021). Compared to conventional animal and plant-derived feed ingredients, insect meals (IM) as a novel feed ingredient have advantages with their ability to efficiently utilize organic waste, small ecological footprints and nutritional values (Rumpold & Schlüter, 2013; Feng et al., 2019). Dried mealworm larvae meal solid-state fermented with probiotics as an alternative feed additive to growth promoters in animal diets has been came up recently (Islam & Yang, 2017). Thus, IM can contribute to reducing environmental concerns (Quang Tran et al., 2022). Seven species of insect meal, including yellow mealworm (TM) (*Tenebrio molitor*) have been approved by the European Union (EU) for use in aquatic feeds (European Commission, 2017). The use of yellow mealworm larvae meal (TML) in aquaculture has recently drawn attention (Feng et al., 2019).

Besides its nutritional quality (protein content, digestibility, flavor), TML can be a functional feed additive (Hong et al., 2020). However, the high chitin levels (more chitin levels than 1.60 %) in TML may negatively affect feed intake and protein availability and thereby worsen growth performance in fish (Ge et al., 2022). Some studies pointed out that one possible strategy to improve the nutritional value and to create functional feed additives of unusual feed ingredients such TML and black soldier fly larvae for domestic animals is solid state fermentation (SSF) (Islam & Yang, 2017; Mulyono et al., 2019; Luparelli et al., 2022). Solid-state fermented feed ingredients are arranged for balanced nutritive characteristics, using probiotics and water under aerobic or anaerobic environments (Zhang et al., 2021). Among the probiotic bacteria species, *Lactobacilli* (Lactic acid bacteria, LAB) is the most used for TML solid-state fermentation (Islam & Yang 2017).

In poultry science, some researchers claimed that the addition of small amounts of TM full-fat meal as an antibacterial feed additive to broiler diet in an *in vivo* study could modulate the microbiota composition of the gastrointestinal tract and improve growth indices (Benzertiha et al., 2019; 2020; Józefiak et al., 2020). However, there is no knowledge about including defatted TML (DTML) solid-state fermented with probiotic bacteria as a functional feed additive to diet in aquaculture, including aquarium fish. In this context, understanding the effects of the DTML solid-state fermented with probiotics as a feed additive on the gut microbiome using new approaches may contribute to elucidating their role in the healthy growth of fish.

The aquarium sector is subject to ethical debates in terms of including organisms obtained from nature (Tlusty et al., 2013). The species cultured in the aquarium must be ensured from aquaculture in a way that does not have a detrimental effect on natural resources (Evers et al., 2019). In accordance with all these approaches, one of the species that attracts attention in the ornamental fish sector is the guppies (Kowalska et al., 2022). Guppies have become the most popular ornamental fish preferred among hobbyists due to their adaptability to changing environmental and ecological conditions, easily raised and fast reproduced cycles. In addition, guppies are utilized as a model organism in studies involving many fields, such as ecology, evolution, behaviour and feeding experiments (Ahmadniaye Motlagh et al., 2020; Sultana et al., 2022). With these versatile characteristics, guppies have an ever-increasing demand in the industry.

Therefore, we hypothesized that DTML fermented with *Lactobacillus brevis* and *L. plantarum* may modulate intestinal microbiota and histomorphometric parameters, improving guppy growth performance; in this study, we report the effects of fermented and non-fermented DTML on growth performance, intestinal microbiota, and histological parameters of the intestine and liver, and body composition in guppies.

Material and Methods

Ethics Statement

The research followed the Animal Experiments Local Ethics Committee guidelines at Ankara University and obtained approval under permission number 2021-11-92.

Experimental Design

The study was performed in the Fisheries Research and Application Unit (FRAU) of the Agricultural Faculty of Ankara University. Total 320 healthy guppies (160 males and 160 females, average body weight of 0.15±0.01 g) in the FRAU were used for the experiment and randomly distributed in 20 fiberglass tanks (45 L) with four replicates of 16 fish each (8 males and 8 females). Four yellow mealworm larva meal diets were formulated to include two probiotics for comparative examination. The yellow mealworm larva meal with fermented and non- fermented was incorporated into the diet as follows: (C) involved with no supplementation; (G1) supplemented with 4 g/kg DTML; (G2) supplemented with 4 g/kg DTML fermented with *L. brevis*; (G3) supplemented with 4 g/kg DTML fermented with *L. plantarum*; (G4) supplemented with combination of 2 g/kg DTML fermented with *L. brevis* plus with 2 g/kg DTML fermented with *L. plantarum.* Physicochemical water quality parameters such as dissolved oxygen (7.51 ±0.06 ppm), temperature (25.27 ±0.52°C, YSI PRO 20, Yellow Springs Instruments) and pH (6.85± 0.11, YSI EcoSense pH10 A, Yellow Springs Instruments) were maintained according to the standard culture conditions of *P. reticulata* (Zdanovich, 2017). Daily 10% water exchange were performed. Continuous aeration and filtration were applied to the tanks via filter pipe. Feeding was applied *ad libitum* twice daily (08:00 AM and 4:00 PM) for 84 days. Natural photoperiod (10L:14D) was adjusted during the experiment.

Preparation of Experimental Diets

TM was acquired from a commercial supplier located in Ankara, Turkey, and was fed with wheat, wheat bran, and carrot. Before processing, TM underwent freeze-drying overnight to eliminate moisture without prior

starvation. The dried TM were ground into the meal with the miller. TM produced from the larval stage of yellow mealworms was full fat. Then, the protein content of TML in the present study was increased by the chemical defatting process since protein may be utilized as substrates by microorganisms for solid-state fermentation (Son et al., 2021). Optimized extraction conditions (petroleum ether to TML ratio of 3:1 L/kg, 60°C for 4 h) with a soxhelet device were applied for defatting of freeze-dried TML. Following the process of defatting, drying of TML was performed at 40°C for 3 h. This process reduced the high-fat content of TML (from 23% to 6.6%) and increased the crude protein content (from 44% to 76.2%).

The protein content, determined based on acid detergent fiber (ADF) according to AOAC (2003), was utilized to calculate the chitin content in defatted TML (DTML) using the procedure outlined by Finke (2007) in the following manner: chitin (%)= ash-free ADF (%)-ADF linked protein (%). The amount of chitin of DTML was 4.2 %. The composition of DTML is shown in Table 1.

For the solid-state fermentation of DTML, two probiotic bacteria (*Lactobacillus plantarum* and *Lactobacillus brevis*) and *Saccharomyces cerevisiae* (baker's yeast) were used. The *Lactobacillus plantarum* strain was isolated from Çeçil cheese, while the *Lactobacillus brevis* strain was isolated from cheddar cheese. *Saccharomyces cerevisiae* (baker's yeast) was produced from sugar beet molasses. *Saccharomyces cerevisiae* during fermentation enhances the viability and growth of lactic acid bacteria (LAB), since it provides some nutrients, such as amino acids and vitamins to LAB (Menezes et al., 2018; Shi et al., 2020). The probiotics used in fermentation, which have chitinase activity, were obtained from Neslihan Dikbaş Microorganism Culture Collection at the Agricultural Biotechnology Laboratories of Ataturk University. Chitinase enzyme activities of the probiotics were analysed in Agricultural Biotechnology Laboratory of Ataturk University (Senol et al., 2014). Chitinase enzyme activity was measured using a colloidal chitin substrate. After adding the substrate to the enzyme solution medium, it was incubated at 37°C for 30 minutes to allow the reaction to occur. Subsequently, staining solutions were added to the reaction mixture, which was then left to stand at 80°C for 10 minutes. Finally, the activity was determined by measuring the absorbance change at 540 nm with a spectrophotometer (PG-T80 Instrument UV–VIS Spectrophotometer) against a blank sample prepared with distilled water. The chitinase enzyme activities of *L. plantarum* and *L. brevis* were found as 15.00 U/L and 11.36 U/L, respectively. The method of Islam & Yang, (2017) was modified in Semi-Solid Phase Fermenter for fermentation of DTML with two different probiotic bacteria in the laboratory of Isparta University of Applied Sciences, Agricultural Faculty, Department of Animal Science.

During the fermentation process of DTML, probiotics were cultivated on solid media consisting of distiller's dried grains with solubles (DDGS) and defatted rice bran. DTML, DDGS, defatted rice bran and water used for fermentation were autoclaved (at 121°C for 15 min) for sterilization. For the fermentation, a mixture including 30 % DTML, 35 % DDGS, 35 % defatted rice bran and 80 % distilled water was prepared. After DDGS, defatted rice bran, DTML and distilled water were placed in the fermenter, carbon dioxide adding was performed to create an anaerobic environment. Incubated *L. plantarum* (100 ml) was added to the medium and performed fermentation under anaerobic conditions (38ºC for 48 h). At the end of this time, *S. cerevisiae* with 1.0 % was used in the second fermentation under anaerobic conditions. Activation of *S. cerevisiae* was performed for 1h at 37ºC in 250 ml of 0.1 % peptone water (10 g yeast + 90 ml peptone water) at 38ºC. After a total of 96 hours completed, the fermented product was dried until less than 15% moisture was achieved at 32ºC for 24 h using a drying oven (Memmert GmbH + Co. KG, Beuchenbach, Germany). The same protocol was carried out for *L. brevis*. To identify microbial concentration, diluted with sterile saline (9 ml of 0.85 %) 1 g of the fermented DTML with *L. plantarum* (FDTMLP) or fermented DTML with *L. brevis* (FDTMLB) were mixed. Total mesophilic aerobic bacteria count was then performed by plating serial 10-fold dilutions in triplicate on Plate Count Agar (PCA) and incubated for 48 hours at 30°C under aerobic conditions. For the *Lactobacillus* counts, it was performed by plating serial 10-fold dilutions in triplicate on De-Man Rogosa and Sharp (MRS) agar and incubated for 5 d at 39-40°C under anaerobic conditions. Yeast and mold counts were made by plating serial 10-fold dilutions in triplicate on Dichloran Rose Bengal Chloramphenicol (DRBC) agar and incubated at 25°C for 5 d under anaerobic conditions. Following the incubation, microbial colonies immediately counted were stated as log10 CFU/g. For FDTMLP and FDTMLB, the nutrient composition and concentrations of microorganisms were presented in Table 2. The protein concentration in association with acid detergent fibre (ADF) was calculated (AOAC, 2003) and used to predict chitin level in FDTMLP and FDTMLB (Finke, 2007) as follows: chitin (%)= ash free ADF (%)-ADF linked protein (%). The chitin amounts of FDTMLP and FDTMLB were 2.74 and 2.81 %, respectively.

Table 1. The nutrients composition of DTML

Nutrient Composition (%)	
Dry Matter	95.70
Crude Protein	76.20
Crude Fat	6.60
Crude Ash	7.30
Starch	3.30
Total Sugar	0.50
Metabolisable Energy, Kcal kg^{-1}	3515

Table 2. The nutrients composition and concentrations of microorganisms in FDTMLP and FDTMLB

Item	FDTMLP	FDTMLB
Microorganisms' concentrations (log10 cfu/g)		
Total Mesophilic Aerobic Bacteria	3.92	4.29
Lactobacillus	2.99	2.45
Yeast-Mold	Not detected	No detected
Nutrient Composition (%)		
Dry Matter	92.00	89.81
Crude Protein	49.28	49.06
Crude Fat	8.14	9.40
Crude Ash	7.81	7.83
Starch	3.73	1.44
Total Sugar	0.36	0.36
Metabolizable Energy, Kcal/kg	2650	2660

Table 3. Ingredients and chemical composition of experimental diets (g kg^{-1} diet)

Ingredients (g kg^{-1})	C	G1	G2	G3	G4
Fish meal	300	300	300	300	300
Soybean meal	130	130	130	130	130
Corn gluten	140	136	136	136	136
Wheat flour	365	365	365	365	365
Fish oil	25	25	25	25	25
Vitamin and mineral premix	10	10	10	10	10
Binder	30	30	30	30	30
Supplement	0	4	4	4	4
Total	1000	1000	1000	1000	1000
Proximate Composition					
Crude protein	380.75	381.20	380.11	380.12	380.14
Crude lipid	79.65	79.67	79.79	79.73	79.75
Crude ash	42.95	43.16	43.18	43.18	43.20

Vit amounts/453 g Vitamin Premix (Vit A: 325,000 USP Units, Vit D3: 65 USP Units, Vit E: 32 USP Units, Vit K 793.65 mg, Vit B12: 10.08 mg, Riboflavin: 3.250 mg, p-Panthothenic acid: 15.600, Niacin: 19.500 mg, Cholin: 2.600 mg, Thiamine: 2.600 mg, Pridoxine Folic acid: 780 mg, Ascorbic acid: 87.100 mg Biotin: 40 mg, BHT: 2 mg, Inositol: 13. Minerals; Manganese 60 ppm, Zinc 50 ppm, Iron 40 ppm, Copper 4 ppm, Cobalt 0.5 ppm, Iodine 40 ppm, Selenium 0.4 ppm (Formulated and Packaged By Florida Aqua Farms Inc. Dade City, Florida).

Sample Collection and Growth Performance

A digital meter (with an accuracy of 0.01 g) was utilized to assess growth parameters (individual body weight of fish from each tank). After a 24-h fasting period, fish (four fish were sampled for each replicate, N = 16) were euthanized with a high dose of clove oil (50 mg/L) to determine the intestinal microbial diversity and body composition. Two fish from each replicate slaughtered were sampled for histological examination of liver and intestinal tissue. Growth parameters were evaluated separately since there was a difference in growth between male and female guppies, but other parameters were evaluated together since they were realized under the same experimental conditions for each group. Growth indices were calculated as follows:

$$WG = FBW - IBW \quad (1)$$

$$SGR = ((ln\ (FBW) - ln\ (IBW))/days) \times 100 \quad (2)$$

$$FCR = \text{Total Given Feed (g)}/WG\ (g) \quad (3)$$

$$SR = (NLE)/(NLB) \times 100 \quad (4)$$

WG : Weight gain (g/fish)
FBW: Final body weight (g)
IBW : Initial body weight (g)
SGR : Specific growth rate (SGR; %/day)
FCR : Feed conversion ratio (FCR)
SR : Survival rate (SR%)
NLE : number of live fish at the end of experiment
NLB : number of live fish at the beginning of the experiment

Intestinal Microbiota

To describe the gut microbiota, the intestinal content was collected from each fish. Samples were taken in the aseptic conditions and then gathered in sterile Eppendorf tubes. Until DNA extraction, all samples were stored at -80°C. For DNA extraction from intestinal contents, it was used the Qiagen Dneasy Blood and Tissue Kit (Spens et al., 2016). After this step, Qubit (Thermo Fisher Scientific, Waltham, MA, USA) was utilized for QC determination. For amplification, 16S Forward and 16S Forward Reverse coded 16S Universal Eubacterium primers specific to 16S rDNA V3-V4 regions were used.

Relevant primers were selected according to the Klindworth et al. (2013) protocol. A 2-step PCR process was performed in the library preparation. 25 cycles of PCR were carried out that used KAPA HiFi HotStart ReadyMix (Roche) to each sample separately for these processes. Following PCR, all samples were subjected to analysis on a 2% agarose gel to verify band presence and assess relative band densities. For measuring the library, Qubit fluorometry was used, and the library was sequenced after normalization. Illumina's 16S Metagenomic Sequencing Library Preparation document include the details of this protocol (Illumina, 2020). The SILVA project's amplicon analysis pipeline was used to process all sequence reads (SILVAngs 1.4) (Quast et al., 2013).

Identification of identical reads, clustering of the unique reads (OTUs) on a per sample basis, and classification of the reference read of each OTU were performed after these first step of quality control (dereplication). For dereplication and clustering, identity criteria of 1.00 and 0.98 were applied,

respectively, and using VSEARCH (version 2.17.0; https: //github.com/torognes/vsearch) (Rognes et al., 2016). The non-redundant version of the SILVA SSU Ref dataset (release 138.1; http://www.arb-silva.de) was employed for classification using BLASTn (2.11.0+; http://blast.ncbi.nlm.nih.gov/Blast.cgi), as outlined by Camacho et al. (2009).

All reads assigned to the respective OTU were mapped to the classification of each OTU reference read. Reads that did not have any or weak classification for which the function "(% sequence ID + % alignment coverage)/2" did not exceed 93 remained unclassified. These reads were defined as the "No Relative" meta group (Ondov et al., 2011).

Histological Analysis

Fish were deeply anaesthetized with clove oil (50.0 mg/L). The liver and intestinal tissues of the slaughtered fish were removed and placed in labeled histological tissue processing cassettes, and the tissues in the cassettes were fixed in 10% formaldehyde using the standard histological methodology. Tissues were embedded in paraffin blocks for histological examination. Sections (5 µ, Shandon AS-325 retraction microtome) were mounted on marked slides before staining with Mayers Haematoxilen and Eosine. Sections were examined and photographed under light trinocular (Leica CM40, Germany) microscopy.

Body Composition

Dry matter and crude ash levels of whole-body in guppies were determined according to AOAC (2003). Crude protein was performed by Kjeldahl method (AOAC, 2003) and crude lipid were analyzed by automated extraction method ANKOM XT-15 (Macedon, NY).

Statistical Analysis

For statistical evaluation, SPSS version 17.0 was used and collected data was given as Mean ± SD. Statistics was performed using one-way ANOVA. Determining differences among the groups was performed through Duncan's multiple range test ($P< 0.05$).

Results and Discussion

For male and female guppies, growth performance parameters, including final weight, weight gain, feed conversion ratio, specific growth rate and survival rate, are given in Table 4. G4 diet significantly ($P<0.001$) increased the final body weight, weight gain and specific growth rate of female guppies compared to the other diets. As shown in Table 4, feeding with the G4 diet significantly ($P<0.05$) improved the FCR of female guppies compared to the G1 diet. The survival rate of female guppies fed the G4 diet was significantly ($P<0.05$) higher than the C, G1 and G2 except for the G3 diet. Feeding with the G3 and G4 diets resulted in higher growth parameters ($P<0.001$) than those of male guppies fed the C, G1 and G2 diets. FCR of male guppies was significantly ($P<0.001$) improved with the G3 and G4 diets compared to that of male guppies fed the C and G1 diets. In addition, the G3 and G4 diets significantly ($P<0.05$) improved the survival rate of male guppies compared to the C and G1 diets, except for the G2 diet,

The bacterial diversity of intestinal digesta samples of the experimental groups is presented in the phylum level in Figure 1. Fusobacteria was the most abundant phylum in C, G1, G2 and G3 groups at the rate of 77, 55, 72 and 37 %, respectively, while Proteobacteria were the second most abundant phylum among these groups as 22, 43, 26 and 31 %, respectively. However, Proteobacteria showed the most intensity in the G4 group, with a rate of 88%. Bacteroidota was found as the second most abundant phylum in the G4 group (6%).

The taxonomic distribution of the bacterial diversity of intestinal digesta samples of the experimental groups at phylum, class, order, family and genus level is detailed in Figures 2-6. In C group, *Cetobacterium* (77%), *Aeromonas* (10%), *Shewanella* (5%), *Pseudomonas* (1%); in G1 group, *Cetobacterium* (55%), *Aeromonas* (26%), *Shewanella* (6%); In G2 group, *Cetobacterium* (72%), *Aeromonas* (12%), *Shewanella* (6%), *Flavobacterium* (1%); In G3 group, *Cetobacterium* (37%), *Nocardia* (19%), *Pseudomonas* (4%), *Shewanella* (5%), *Aeromonas* (2%), *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (2%), *Kaistia* (2%), *Rhodococcus* (2%), *Neochlamydia* (2%), *Lysinimonas* (1%), *Mycobacterium* (1%), *Flavobacterium* (1%), *Fimbriiglobus* (1%); In G4 group, *Aeromonas* (20%), *Shewanella* (12%), *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (12%), *Comanonas* (11%), *Flavobacterium* (5%), *Bosea* (3%), *Shinella* (2%), *Kaistia* (1%), *Cetobacterium* (1%) were abundant at genus level..

Table 4. The effects yellow mealworm (*Tenebrio molitor*) larvae meal on growth performance of female and male guppies

		C	G1	G2	G3	G4	*p value*
Initial body weight (g)	Female	0.15±0.01	0.15±0.01	0.14±0.01	0.15±0.01	0.15±0.01	0.087
	Male	0.15±0.01	0.15±0.01	0.15±0.01	0.15±0.01	0.15±0.01	0.959
Final body weight (g)	Female	1.25±0.09^{c}	1.21±0.11^{cd}	1.18±0.10^{d}	1.54±0.08^{b}	1.66±0.09^{a}	0.000
	Male	0.49±0.05^{b}	0.51±0.07^{b}	0.41±0.04^{c}	0.64±0.03^{a}	0.64±0.04^{a}	0.000
Body weight gain (g)	Female	1.10±0.07^{c}	1.06±0.05^{c}	1.04±0.03^{c}	1.39±0.04^{b}	1.52±0.03^{a}	0.000
	Male	0.34±0.03^{b}	0.37±0.03^{b}	0.26±0.03^{c}	0.49±0.02^{a}	0.49±0.03^{a}	0.000
SGR (%)	Female	2.53±0.08^{c}	2.48±0.08^{c}	2.52±0.05^{c}	2.77±0.05^{b}	2.88±0.02^{a}	0.000
	Male	1.42±0.07^{b}	1.48±0.07^{b}	1.21±0.09^{c}	1.75±0.04^{a}	1.75±0.07^{a}	0.000
Survival rate (%)	Female	92.19±3.13^{b}	92.19±3.13^{b}	90.63±3.61^{b}	95.31±3.13^{ab}	98.44±3.13^{a}	0.026
	Male	89.06±3.13^{b}	89.06±3.13^{b}	92.19±3.13^{ab}	95.31±3.13^{a}	96.88±3.61^{a}	0.010
FCR	Female	1.65±0.02^{ab}	1.67±0.05^{a}	1.65±0.03^{ab}	1.65±0.02^{ab}	1.61±0.02^{b}	0.012
	Male	1.87±0.04^{a}	1.78±0.13^{ab}	1.69±0.06^{bc}	1.56±0.03^{d}	1.61±0.04^{cd}	0.000

Values are mean ± SD. Means in the same raw text with different superscripts are significantly different

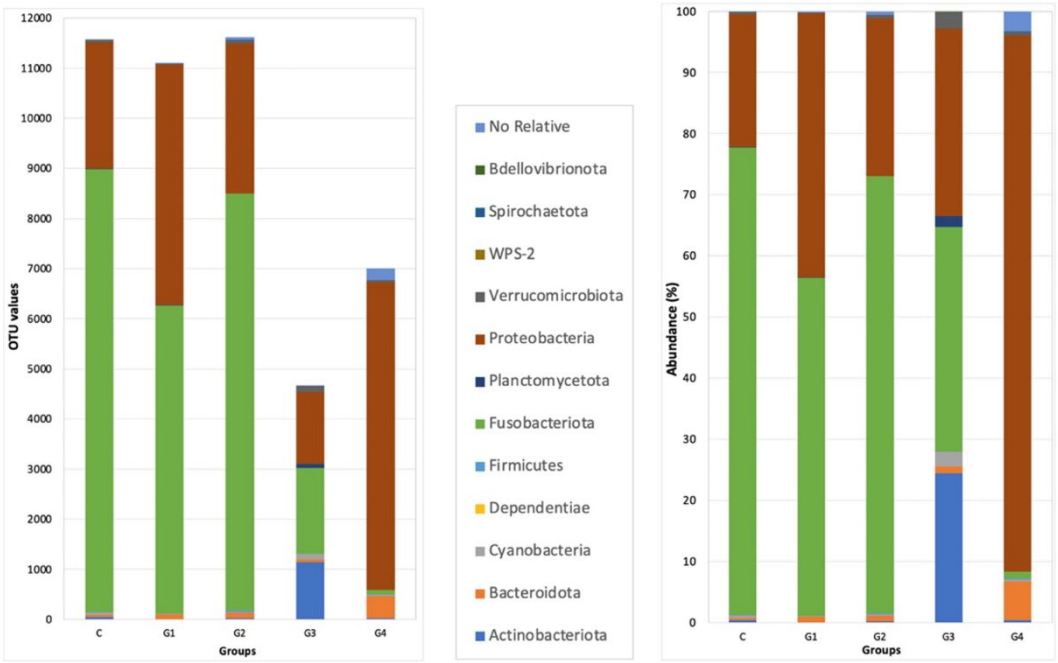

Figure 1. Relative abundance (%) and OTU values of the most prevalent bacteria among the groups at the phylum taxonomic level

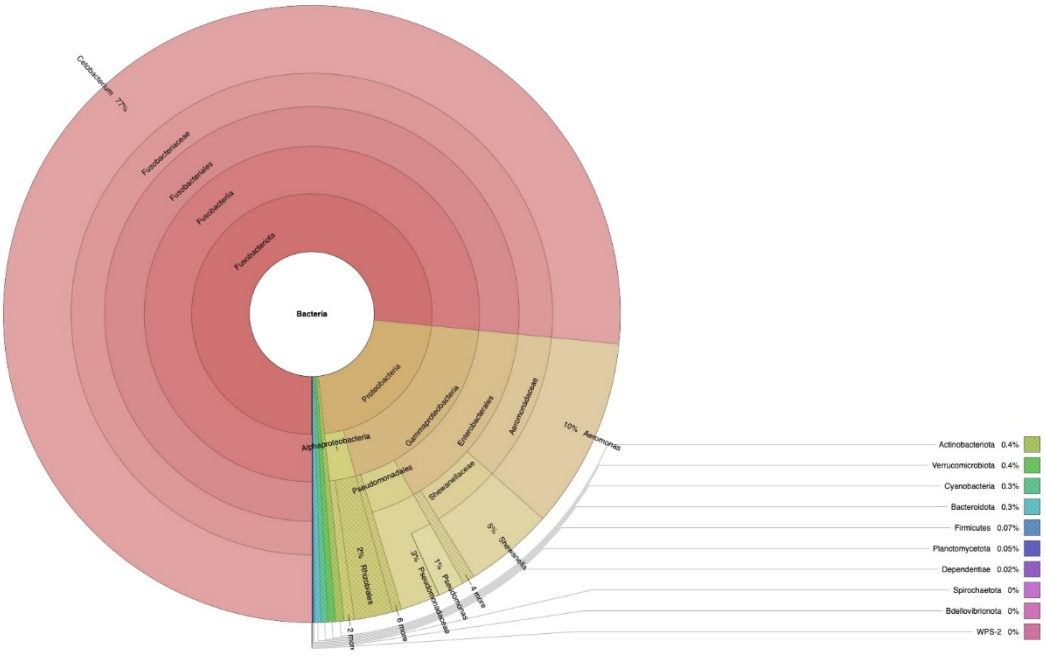

Figure 2. Krona graph of C group showing microbial population in intestinal digesta samples of guppies

Histopathological analysis of intestinal and liver tissue were compared to distinguish the effect of different feed additives in guppy fish (Figure 7). Irregular-shaped hepatocytes were seen in histological sections of the liver tissue. In general, however, tissue with a typical cord-like arrangement was identified, surrounded by hepatocytes, sinusoids, and connective tissues. In addition, lipid accumulation was found in hepatocytes, and the presence of lipoid vacuoles (as glycogen stored) in the liver was compatible with using the formulated diet. The numerous lipid vacuoles observed in hepatocytes in liver tissues of guppy showed regular and moderate infiltrations. The hepatic parenchyma was detected as sponge-like morphology, polyhedral type hepatocytes, and lipid droplets for all groups.

It is the appearance of intestinal folds, and in the histopathological examination of the intestine, it was noted that the first inflammatory reactions such as ciliary epithelium and well-distributed goblet structure and lamina propria and lamina epithelial hyperplasia were not observed. Furthermore, no remarkable difference was determined in goblet cell densities. It is seen that the use of these feed additives in guppy culture exhibited normal histomorphology in the liver and small intestine tissues.

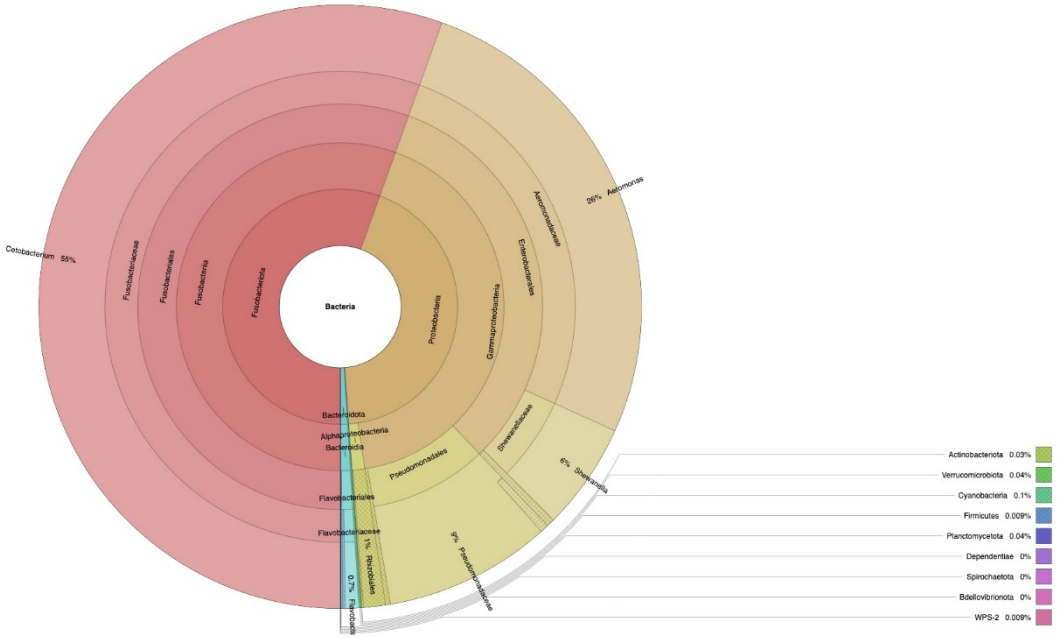

Figure 3. Krona graph of G1 group showing microbial population in intestinal digesta samples of guppies

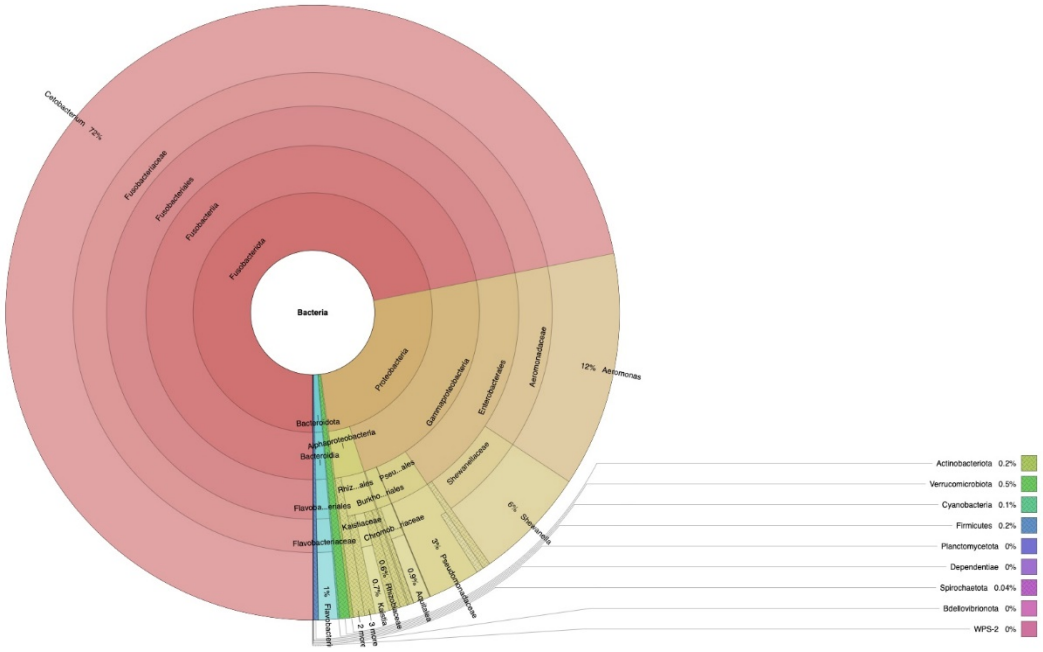

Figure 4. Krona graph of G2 group showing microbial population in intestinal digesta samples of guppies

The effects yellow mealworm (*T. molitor*) larvae meal on histomorphometric parameters of the intestine of guppies are summarized in Table 5. G4 diet had significantly ($P<0.05$) the highest villus height of the small intestine of guppies. Small intestinal villus width of guppies had the highest value in fish fed with G1 and G3 rations ($P<0.01$). Moreover, feeding with the G3 and G4 diet significantly increased the surface absorption area of the small intestine of guppies compared to feeding with other diets ($P<0.05$). Feeding with G2, G3 and G4 diets significantly reduced the crypt depth of the small intestine of guppies compared to feeding with C and G1 diets ($P<0.05$). No significant differences were observed among the groups regarding moisture, protein, lipids and ash contents of the whole body in guppies (Table 6).

Among the alternative ingredients for aquafeed, TM is one of the promising candidates in the aquaculture industry (Tran et al., 2022). The utilization of TM for partial/complete replacement of protein sources in aquafeed has been studied in detail for most cultured fish species, including yellow catfish (*Pelteobagrus fulvidraco*) (Su et al., 2017), Nile tilapia (*Oreochromis niloticus*) (Sánchez-Muros et al., 2016), rainbow trout (*Oncorhynchus mykiss*) (Chemello et al., 2020; Jeong et al., 2020), European sea bass (*Dicentrarchus labrax*) (Henry et al., 2018; Mastoraki et al., 2020; Basto et al., 2020), gilthead sea bream (*Sparus aurata*) (Piccolo et al., 2017), Atlantic salmon (*Salmo salar*) (Biancarosa et al., 2019), and red seabream (*Pargus major*) (Ido et al., 2019).

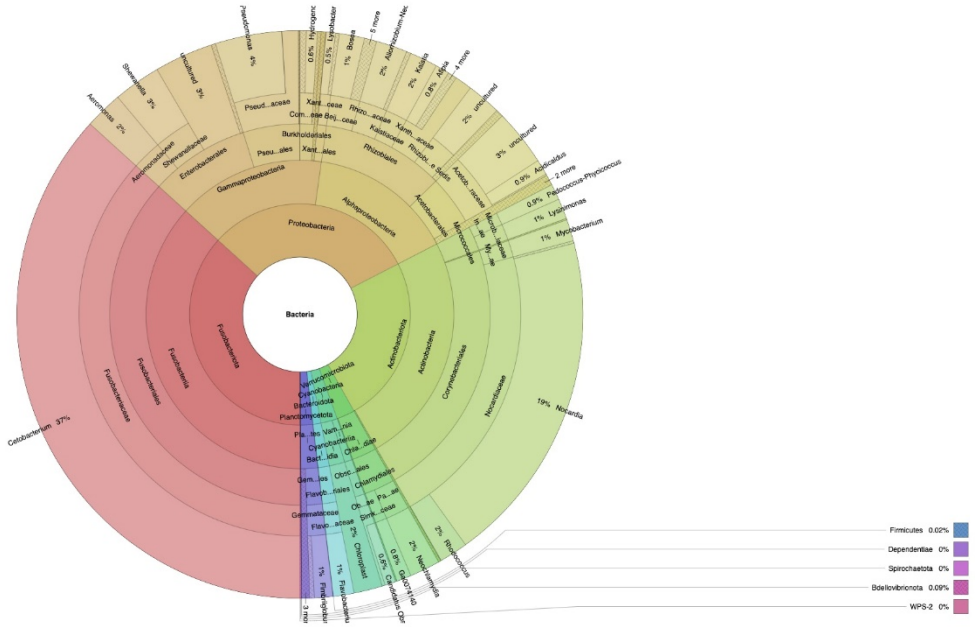

Figure 5. Krona graph of G3 group showing microbial population in intestinal digesta samples of guppies

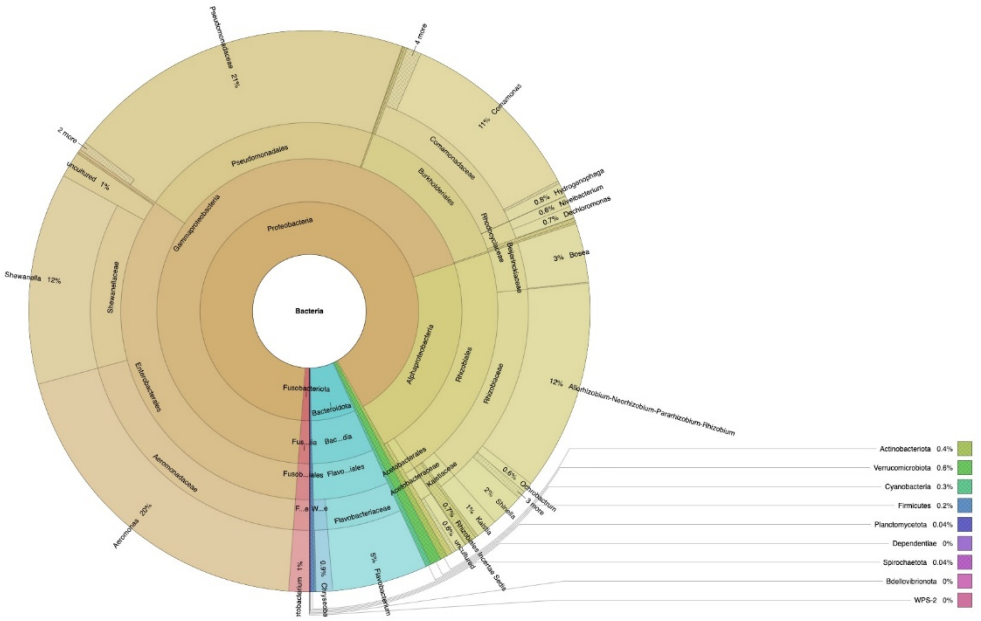

Figure 6. Krona graph of G4 group showing microbial population in intestinal digesta samples of guppies

Table 5. The effects yellow mealworm (*T. molitor*) larvae meal on histomorphometric parameters of intestine of guppies

Histomorphometric parameters	C	G1	G2	G3	G4	p value
Villus height, µm	138.80±26.90^{d}	151.20±41.74^{c}	159.40±66.06^{c}	184.40±31.50^{b}	194.40±34.07^{a}	0.024
Villus width, µm	36.60±11.24^{c}	60.80±11.63^{a}	53.00±1.83^{b}	64.00±10.25^{a}	57.80±5.68^{b}	0.001
Villus surface area, µm^2	5.13±2.07^{c}	9.47±3.80^{b}	10.17±4.40^{b}	11.32±3.04^{a}	11.09±1.16^{a}	0.033
Crypt depth, µm	4.80±0.84^{a}	4.60±0.65^{a}	3.70±1.04^{b}	3.60±0.42^{b}	3.20±1.04^{b}	0.028

Values are mean ± SD. Means in the same raw text with different superscripts are significantly

Table 6. Nutrient composition of whole-body on a wet weight basis (%)

	C	G1	G2	G3	G4	p value
Moisture	77.10±0.30	77.53±0.23	77.00±0.36	77.07±0.29	76.73±0.2	0.066
Lipids	3.30±0.36	3.60±0.17	3.37±0.15	3.37±0.51	3.27±0.32	0.761
Protein	13.73±0.21	13.87±0.38	14.03±0.42	14.03±0.15	14.10±0.00	0.508
Ash	3.63±0.15	3.47±0.12	3.60±0.10	3.57±0.21	3.50±0.17	0.673

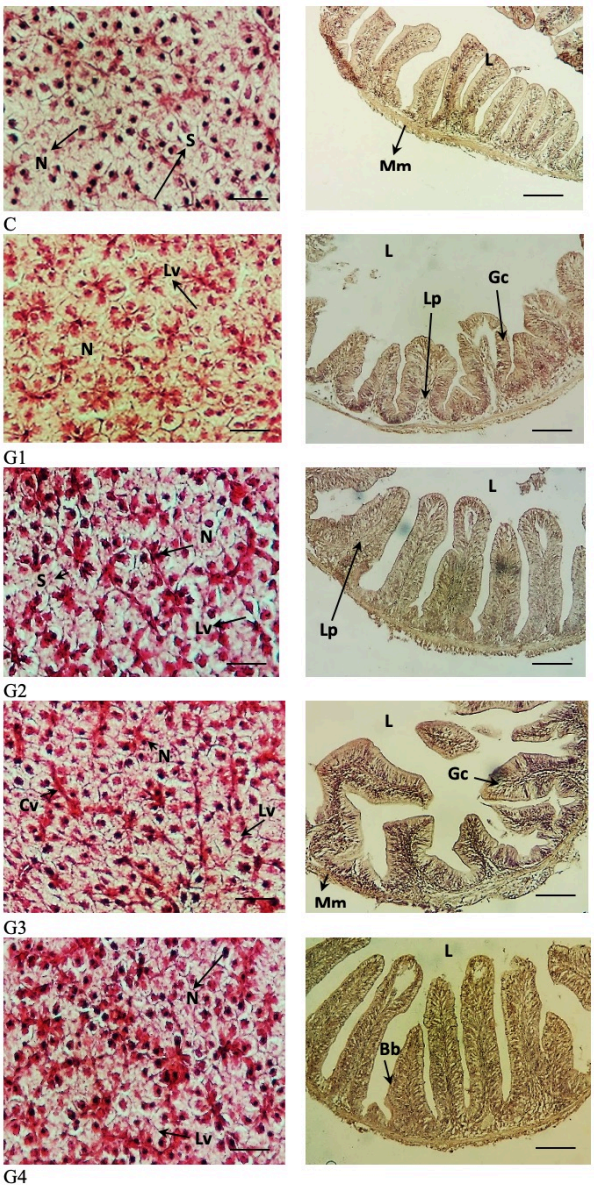

Figure 7. The liver (left column) and small intestine (right column) histology sections of guppies fed with the non-fermented and fermented Tenebrio molitor base meals.
N: hepatocyte nucleus, Lv: lipoid vacuoles, S: sinusoids, and C: capillary vessels, Gc: goblet cell, Lp: lamina propria, Bb: brush border, L: lumen, Mm: muscularis mucosa (H&E, x400, bar: 50 µm).

Although TM is not only considered as a substitution material but also as a functional feed additive, there is no information on its use as a feed additive in fish culture. This study indicates the first attempt at the possibility of using solid-state fermented DTML as a functional antibacterial feed additive in guppies. In a study conducted by Wang et. al. (2020), the addition of 0.4% solid-state fermented wheat bran polysaccharides to the diet increased the growth performance of common carp compared to the control group. Also, it was reported that the inclusion of 0.5% solid-state fermentation product (Synergen™) to the diet could significantly increase growth performance and feed efficiency of rainbow trout. In the present study, the inclusion of the solid-state fermented DTML with *L. plantarum*, particularly *L. plantarum* plus *L. brevis* as a feed additive, resulted in better growth indices of *P. reticulata* after 84 days. The better growth performance values obtained in the G4 groups can be attributed to the feed additive, which gained functional quality after fermentation with *L. plantarum* and *L. brevis*, by improving the intestinal microbiota and increasing nutrient absorption. *Lactobacillus* spp. and *Saccharomyces* spp. called probiotics are frequently used in animal breeding as a fermentation and feed additive (Wang et al., 2018; Wang & Jin, 2019; Zhang et al., 2021).

Furthermore, combinations of probiotics prove to be more effective in aquaculture compared to using individual probiotic strains (Lin et al., 2017). In parallel with our results, in a study conducted by Zhang et al. (2021), it was concluded that fermented feed with probiotics mixture increased growth indices in *Penaeus vannamei*. According to another previous study by Lin et al. (2017), it was stated that the synergistic effect of the probiotic mixture as a feed additive improved growth performance for *Lates calcarifer*.

The gut microbiome plays a crucial role in improving the digestion of nutrients and promoting healthy fish growth (Ganguly & Prasad, 2012). In some previous studies, it was stated that the inclusion of TM in the diet did not affect the gut microbiota (Mikołajczak et al., 2020; Terova et al., 2021; Jeong et al., 2021). In contrast, in others, it was claimed that the gut microbiota changed significantly (Antonopoulou et al., 2019; Józefiak et al., 2019). However, it was stated that fermented feed with mixed probiotics could improve species richness and evenness of shrimp microbiota (Zang et al., 2021). In our study, while unfermented DTML did not vary the gut microbiota, fermented DTML with *L. plantarum* and *L. brevis* feed additive increased gut microbiota diversity in the guppies. This critical difference among the groups may be due to the synergistic effect of the feed additive fermented with different probiotics in the intestine and the bioactive substances found in the DTML. Contrasting data from the current literature may depend on determinant parameters such as the rearing medium of TM or TML used as a feed ingredient and the quantity of protein and lipid in its body. These differences can also be attributed to the fish species, inclusion levels and nutrient requirements.

Fusobacteria is often identified as the most common phylum in freshwater fish (Roeselers et al., 2011; Ghanbari et al., 2015; Parata et al., 2020). In agreement with the previous data, Fusobacteria were a major constituent of the guppies microbiome in the C, G1, G2 and G3 groups. However, the most prevalent bacteria in the samples of guppies fed fermented with *L. plantarum* plus *L. brevis* feed additive were Proteobacteria phylum. In different studies, it was indicated that the Proteobacteria may differ depending on diet formulation (Ingerslev et al., 2014; Gajardo et al., 2016). This approves that the inclusion of the fermented DTML with two probiotics changed the dominant phylum in the G4 group when compared to the other groups. Within this phylum, bacteria such as *Aeromonas*, *Pseudomonas* and *Shewanella* were increased in the G4 group. Although *Aeromonas* is primarily associated with disease, it is found in the GI tract of healthy finfish (Abdelhamed et al., 2019). There is general information regarding using *Aeromonas* as a probiotic in fish culture (Chi et al., 2014; Wu et al., 2015; Hao et al., 2017). Various studies have also shown the probiotic potential of Pseudomonas (Giri et al., 2011; 2015; 2016). The genus *Shewanella* is used commonly as a probiotic in

finfish culture (Chabrillón et al., 2005a; 2005b). In the present study, it can be stated that fermented probiotic mixture stimulated gut microbiota with beneficial probiotic bacteria.

With the morphometric measurement of intestinal villi height and width, the mechanism of absorption and digestion in the fish intestine can be predicted (Rašković et al., 2011). In our study, the measured intestinal villus height, width, and surface area increased in all experimental groups compared to the control group, reaching the highest value in fish fed DTMLM fermented with *L. plantarum* plus *L. brevis*. The improved feed utilization in fish fed fermented feed additives could be attributed to the increasing values of the intestinal villi, thus improving metabolism and nutrient absorption. Consistent with our study data, villus length of the intestinal sections was enhanced in fish fed yeast fermented PBM in common carp (Dawood et al., 2020b) and in fish fed yeast fermented date palm seed meal in Nile tilapia (Dawood et al., 2020a).

In our study, DTML feed additive did not change the nutrient composition of whole body in guppies. Similar results were obtained in studies in which DTML was included in the diet at different levels (Sankian et al., 2018; Rema et al., 2019; Tran et al., 2022).

In conclusion, the findings of this study compellingly demonstrate that the inclusion of 2 g/kg *L. plantarum* and 2 g/kg *L. brevis* fermented DTML significantly enhances the growth performance of guppies. This improvement is attributed to the positive modulation of gut microbiota and the enhancement of gut histomorphology, offering substantial implications for scientists in the field of aquaculture and aquarium fish industry. However, the inclusion of fermented DTML as a feed additive needs to be tested in further research to identify the mechanisms for cultured fish species.

Declarations

Acknowledgement

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Data Availability

Data will be made available on request.

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